NUCLEIC ACIDS FOR DETECTING *ASPERGILLUS* SPECIES AND OTHER FILAMENTOUS FUNGI

This invention was made in the Centers for Disease Control Mycotic Diseases Laboratories, an agency of the United States Government.

Technical Field

This application relates in general to the field of diagnostic microbiology. In particular, the invention relates to the species-specific detection of *Aspergillus, Fusarium, Mucor, Penicillium, Rhizopus, Rhizomucor, Absidia, Cunninghamella, Pseudallescheria boydii (Scedosporium apiospermum),* and *Sporothrix* species.

Background of the Invention

In recent years, chemotherapy for hematological malignancies, and high-dose corticosteroid treatment for organ transplant recipients, along with the spread of AIDS, have greatly increased the number of immunocompromised patients (1, 12, 14, 43). Saprophytic filamentous fungi, such as *Aspergillus, Rhizopus,* and *Mucor* species, found in the environment and considered to be of low virulence, are now responsible for an increasing number of infections in the immunocompromised host (17, 20, 43). In addition, these infections are often fulminant and rapidly fatal in immunocompromised patients (7, 11, 12, 20, 44). Morbidity and mortality is extremely high; for example, aspergillosis has a mortality rate of approximately 90% (8, 11).

To complicate matters, diagnosis is difficult and symptoms are often non-specific (18, 27, 29, 42, 44). Antibody-based tests can be

unreliable due to the depressed or variable immune responses of immunocompromised patents (2, 9, 18, 46). Antigen detection tests developed to date have fallen short of the desired sensitivity (2, 9, 38). Radiographic evidence can be non-specific and inconclusive (5, 29, 36), although some progress in diagnosis has been made with the advent of computerized tomography (40). However, definitive diagnosis still requires either a positive blood or tissue culture or histopathological confirmation (3, 21). An added complication is that the invasive procedures necessary to obtain biopsy materials are often not recommended in thrombocytopenic patient populations (37, 41).

Even when cultures of blood, lung or rhinocerebral tissues are positive, morphological and biochemical identification of filamentous fungi can require several days for adequate growth and sporulation to occur, delaying targeted drug therapy. Some atypical isolates may never sporulate, making identification even more difficult (23). When histopathology is performed on tissue biopsy sections, the morphological similarities of the various filamentous fungi in tissue make differentiation difficult (16). Fluorescent antibody staining of histopathological tissue sections is not specific unless cross-reactive epitopes are absorbed out which can make the resultant antibody reactions weak (14, 19). Therapeutic choices vary (7, 41, 44) making a test to rapidly and specifically identify filamentous fungi urgently needed for the implementation of appropriately targeted therapy. Early and accurate diagnosis and treatment can decrease morbidity and increase the chances for patient survival (6, 27, 39). Furthermore, identification of filamentous fungi to at least the species level would be epidemiologically useful (24, 31, 43, 47).

PCR-based methods of detection, which show promise as rapid, sensitive means to diagnose infections, have been used in the identification of DNA from *Candida* species (13, 15, 30) and some other fungi, particularly *Aspergillus* species (31, 33, 45). However, most of these tests are only genus-specific (28, 38) or are directed to detect only single-copy genes (4, 35). Others have designed probes to detect multi-copy genes so as to increase test sensitivity (31, 33) but in doing so have lost test specificity because they have used highly conserved genes, which detect one or a few species but which are also plagued with cross-reactivities to human, fungal or even viral DNA (25, 31, 33).

Therefore, it is an object of the invention to provide improved materials and methods for detecting and differentiating *Aspergillus* and other filamentous fungal species in the clinical and laboratory settings.

Summary of the Invention

The present invention relates to nucleic acids for detecting *Aspergillus, Fusarium, Mucor, Penicillium, Rhizopus, Rhizomucor, Absidia, Cunninghamella, Pseudallescheria (Scedosporium),* and *Sporothrix* species. Unique internal transcribed spacer 2 coding regions permit the development of probes specific for five different *Aspergillus* species, *A. flavus, A. fumigatus, A. niger, A. terreus,* and *A. nidulans*. The invention thereby provides methods for the species-specific detection and diagnosis of *Aspergillus* infection in a subject. In addition, species probes have been developed for three *Fusarium*, four *Mucor*, two *Penicillium*, five *Rhizopus* and one *Rhizomucor* species, as well as probes for *Absidia corymbifera, Cunninghamella elegans, Pseudallescheria boydii (Scedosporium apiospermum)*, and *Sporothrix schenckii*. Generic probes for *Aspergillus, Fusarium*, and *Mucor* species have also been developed.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Detailed Description of the Invention

This invention provides a simple, rapid, and useful method for differentiating filamentous fungal species from each other and from other medically important fungi. This invention enables a rapid, simple and useful method to isolate fungal DNA from host samples, and to apply the species- and genus-specific probes for the diagnosis of a disease. Ultimately, these probes can be used for *in situ* hybridization or *in situ* PCR diagnostics so that the morphology of host tissue, and microorganisms, remain intact.

The invention provides nucleic acids containing regions of specificity for five *Aspergillus*, three *Fusarium*, four *Mucor*, two *Penicillium*, five *Rhizopus* and one *Rhizomucor* species as well as probes for *Absidia corymbifera, Cunninghamella elegans, Pseudallescheria boydii* (*Scedosporium apiospremum*), and *Sporothrix schenckii*. These nucleic acids are from the internal transcribed spacer 2 ("ITS2") region of ribosomal deoxyribonucleic

acid (rDNA) of the genome of the aforementioned filamentous fungi. The ITS2 region is located between the 5.8S rDNA region and the 28S rDNA region.

In particular, the invention provides nucleic acids from *Aspergillus flavus* (SEQ ID NO:1), *Aspergillus fumigatus* (SEQ ID NO:2), *Aspergillus niger* (SEQ ID NO:3), *Aspergillus terreus* (SEQ ID NO:4), *Aspergillus nidulans* (SEQ ID NO:5), *Fusarium solani* (SEQ ID NO:6), *Fusarium moniliforme* (SEQ ID NO:7), *Mucor rouxii* (SEQ ID NO:8), *Mucor racemosus* (SEQ ID NO:9), *Mucor plumbeus* (SEQ ID NO:10), *Mucor indicus* (SEQ ID NO:11), *Mucor circinilloides f. circinelloides* (SEQ ID NO:12), *Rhizopus oryzae* (SEQ ID NO:13 and NO:14), *Rhizopus microsporus* (SEQ ID NO:15 and 16), *Rhizopus circinans* (SEQ ID NO:17 and 18), *Rhizopus stolonifer* (SEQ ID NO: 19), *Rhizomucor pusillus* (SEQ ID NO:20), *Absidia corymbifera* (SEQ ID NO:21 and 22), *Cunninghamella elegans* (SEQ ID NO:23), *Pseudallescheria boydii* (teleomorph of *Scedosporium apiospermum*) (SEQ ID NO:24, 25, 26, and 27), *Penicillium notatum* (SEQ ID NO:28), and *Sporothrix schenkii* (SEQ ID NO:29). These sequences can be used to identify and distinguish the respective species of *Aspergillus, Fusarium, Mucor, Rhizopus,* and *Penicillium,* and identify and distinguish these species from each other and from *Absidia corymbifera, Cunninghamella elegans, Pseudallescheria boydii(Scedosporium apiospermum),* and *Sporothrix schenkii.*

Furthermore, the invention provides isolated nucleic acid probes derived from GenBank nucleic acid sequences (for *Penicillium marneffei* and *Fusarium oxysporum* only) or from the above nucleic acid sequences which may be used as species-specific identifiers of *Aspergillus flavus* (SEQ ID NO:30 and 31), *Aspergillus fumigatus* (SEQ ID NO:32), *Aspergillus niger* (SEQ ID NO:33), *Aspergillus terreus* (SEQ ID NO:34), *Aspergillus nidulans* (SEQ ID NO: 35), *Mucor rouxii* (SEQ ID NO:36), *Mucor plumbeus* (SEQ ID NO:37), *Mucor indicus* (SEQ ID NO:38), *Mucor circinilloides f. circinelloides* (SEQ ID NO:39), *Mucor racemosus* (SEQ ID NO:40), *Rhizopus oryzae* (SEQ ID NO:41), *Rhizopus circinans* (SEQ ID NO:42), *Rhizomucor pusillus* (SEQ ID NO:43), *Rhizopus stolonifer* (SEQ ID NO:44), *Pseudallescheria boydii* (*Scedosporium apiospermum*)(SEQ ID NO:45), *Penicillium notatum* (SEQ ID NO:46), *Penicillium marneffei* (SEQ ID NO:47 and 48), *Fusarium moniliforme* (SEQ ID NO:49), *Fusarium oxysporum* (SEQ ID NO:50), *Fusarium solani* (SEQ ID NO:51),

Cunninghamella elegans (SEQ ID NO: 52, 53, and 54), *Absidia corymbifera* (SEQ ID NO:55), *Sporothrix schenkii* (SEQ ID NO:56), and *Rhizopus microsporus* (SEQ ID NO:57). Such probes can be used to selectively hybridize with samples containing nucleic acids from species of *Aspergillus, Fusarium, Mucor, Rhizopus* (or *Rhizomucor*), *Penicillium,* or from *Absidia corymbifera, Cunninghamella elegans, Pseudallescheria boydii* (*Scedosporium apiospermum*), and *Sporothrix schenkii.* These fungi can be detected after polymerase chain reaction or ligase chain reaction amplification of fungal DNA and specific probing of amplified DNA with DNA probes labeled with digoxigenin, reacted with anti-digoxigenin antibodies labeled with horseradish peroxidase and a colorimetric substrate, for example. Additional probes can routinely be derived from the sequences given in SEQ ID NOs:1-29, which are specific for the respective species. Therefore, the probes shown in SEQ ID NOs:30-57 are only provided as examples of the species-specific probes that can be derived from SEQ ID NOs:1-29.

Generic probes for *Aspergillus* (SEQ ID NO:58), *Fusarium*, (SEQ ID NO:59) and *Mucor* (SEQ ID NO:60) species have also been developed to identify all members of their respective species which are listed above as well as an all-fungus biotinylated probe (SEQ ID NO:61) to capture all species-specific and generic probes listed above for their detection.

By "isolated" is meant nucleic acid free from at least some of the components with which it naturally occurs. By "selective" or "selectively" is meant a sequence which does not hybridize with other nucleic acids to prevent adequate determination of an *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus* or *Rhizomucor* genus or species or of *Absidia corymbifera, Cunninghamella elegans, Pseudallescheria boydii (Scedosporium apiospermum)*, or *Sporothrix schenckii* species.

The hybridizing nucleic acid should have at least 70% complementarity with the segment of the nucleic acid to which it hybridizes. As used herein to describe nucleic acids, the term "selectively hybridizes" excludes the occasional randomly hybridizing nucleic acids and thus has the same meaning as "specifically hybridizing". The selectively hybridizing nucleic acids of the invention can have at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, and 99% complementarity with the segment of the sequence to which it hybridizes.

The invention contemplates sequences, probes and primers which selectively hybridize to the complementary, or opposite, strand of DNA

as those specifically provided herein. Specific hybridization with nucleic acid can occur with minor modifications or substitutions in the nucleic acid, so long as functional species-specific or genus-specific hybridization capability is maintained. By "probe" is meant nucleic acid sequences that can be used as probes or primers for selective hybridization with complementary nucleic acid sequences for their detection or amplification, which probes can vary in length from about 5 to 100 nucleotides, or preferably from about 10 to 50 nucleotides, or most preferably about 18 nucleotides. The invention provides isolated nucleic acids that selectively hybridize with the species-specific nucleic acids under stringent conditions and should have at least 5 nucleotides complementary to the sequence of interest. *See generally, Maniatis (26).*

If used as primers, the invention provides compositions including at least two nucleic acids which hybridize with different regions so as to amplify a desired region. Depending on the length of the probe or primer, target region can range between 70% complementary bases and full complementarity and still hybridize under stringent conditions. For example, for the purpose of diagnosing the presence of the *Aspergillus*, the degree of complementarity between the hybridizing nucleic acid (probe or primer) and the sequence to which it hybridizes (e.g., *Aspergillus* DNA from a sample) is at least enough to distinguish hybridization with a nucleic acid from other yeasts and filamentous fungi. The invention provides examples of nucleic acids unique to each filamentous fungus in the listed sequences so that the degree of complementarity required to distinguish selectively hybridizing from nonselectively hybridizing nucleic acids under stringent conditions can be clearly determined for each nucleic acid.

Alternatively, the nucleic acid probes can be designed to have homology with nucleotide sequences present in more than one species of the fungi listed above. Such a nucleic acid probe can be used to selectively identify a group of species such as the generic probes listed for *Aspergillus* (SEQ ID NO:58), *Fusarium* (SEQ ID NO:59), and *Mucor* (SEQ ID NO:60) as well as all fungi listed (SEQ ID NO:61). Additionally, the invention provides that the nucleic acids can be used to differentiate the filamentous fungi listed in general from other filamentous fungi and yeasts, such as *Candida* species. Such a determination is clinically significant, since therapies for these infections differ.

The invention further provides methods of using the nucleic acids to detect and identify the presence of the filamentous fungi listed, or

particular species thereof. The method involves the steps of obtaining a sample suspected of containing filamentous fungi. The sample may be taken from an individual, such as blood, saliva, lung lavage fluids, vaginal mucosa, tissues, etc., or taken from the environment. The filamentous fungal cells can then be lysed, and the DNA extracted and precipitated. The DNA is preferably amplified using universal primers derived from the internal transcribed spacer regions, 18S, 5.8S and 28S regions of the filamentous fungal rDNA. Examples of such universal primers are shown below as ITS1 (SEQ ID NO: 62), ITS3 (SEQ ID NO: 63), ITS4 (SEQ ID NO: 64). Detection of filamentous fungal DNA is achieved by hybridizing the amplified DNA with a species-specific probe that selectively hybridizes with the DNA. Detection of hybridization is indicative of the presence of the particular genus (for generic probes) or species (for species probes) of filamentous fungus.

Preferably, detection of nucleic acid (e.g. probes or primers) hybridization can be facilitated by the use of detectable moieties. For example, the species-specific or generic probes can be labeled with digoxigenin, and an all-fungus probe, such as described in SEQ ID NO:61, can be labeled with biotin and used in a streptavidin-coated microtiter plate assay. Other detectable moieties include radioactive labeling, enzyme labeling, and fluorescent labeling, for example.

The invention further contemplates a kit containing one or more species-specific probes, which can be used for the detection of particular filamentous fungal species and genera in a sample. Such a kit can also contain the appropriate reagents for hybridizing the probe to the sample and detecting bound probe. The invention may be further demonstrated by the following non-limiting examples.

Examples

In this example, PCR assay employing universal, fungus-specific primers and a simple, rapid EIA-based format for amplicon detection were used.

Extraction of Filamentous Fungal DNA

A mechanical disruption method was used to obtain DNA from filamentous fungal species and an enzymatic disruption method described previously (13) was used to obtain DNA from yeasts. Filamentous fungi were grown for 4 to 5 days on Sabouraud dextrose agar slants (BBL,

division of Becton Dickinson, Cockeysville, MD) at 35°C. Two slants were then washed by vigorously pipeting 5 mls of 0.01 M potassium phosphate buffered saline (PBS) onto the surface of each slant and the washes were transferred to 500 ml Erlenmeyer flasks containing 250 ml of Sabouraud dextrose broth (BBL). Flasks were then incubated for 4 to 5 days on a rotary shaker (140 rpm) at ambient temperature. Growth was then harvested by vacuum filtration through a sterile Whatman #1 filter paper which had been placed into a sterile Buchner funnel attached to a 2 L side-arm flask. The resultant cellular mat was washed on the filtration apparatus three times with sterile distilled water, removed from the filter paper by gentle scraping with a rubber policeman, and placed into a sterile Petri plate which was then sealed with parafilm and frozen at -20°C until used.

Just prior to use, a portion of the frozen cellular mat, equal in size to a quarter, was removed and placed into a cold mortar (6" diameter). Liquid nitrogen was added to cover the mat which was then ground into a powder with a pestle. Additional liquid nitrogen was added as needed to keep the mat frozen during grinding.

DNA was then purified using proteinase K and RNase treatment, multiple phenol extractions, and ethanol precipitation by conventional means (26).

PCR amplification

The fungus-specific, universal primer pair ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') (SEQ ID NO: 63) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (SEQ ID NO: 64) was used to amplify a portion of the 5.8S rDNA region, the entire ITS2 region, and a portion of the 28S rDNA region for each species as previously described (13, 34). DNA sequencing used this primer pair and also the fungus-specific, universal primer pair ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') (SEQ ID NO: 62) and ITS4 to amplify a portion of the 18S rDNA region, the entire 5.8S region, the entire ITS1 and ITS2 regions, and a portion of the 28S rDNA region.

A DNA reagent kit (TaKaRa Biomedicals, Shiga, Japan) was used for PCR amplification of genomic DNA. PCR was performed using 2 µl of test sample in a total PCR reaction volume of 100 µl consisting of 10 µl of 10X Ex Taq buffer, 2.5 mM each of dATP, dGTP, dCTP, and dTTP, in 8 µl, 0.2 µM of each primer, and 0.5 U of TaKaRa Ex Taq DNA polymerase.

Thirty cycles of amplification were performed in a Perkin-Elmer 9600 thermal cycler (Emeryville, CA) after initial denaturation of DNA at 95°C for 5 minutes. Each cycle consisted of a denaturation step at 95°C for 30 seconds, an annealing step at 58°C for 30 seconds, and an extension step at 72°C for 1 minute. A final extension at 72°C for 5 minutes followed the last cycle. After amplification, samples were stored at -20°C until used.

Table 1
Synthetic Universal Oligonucleotides Used in PCR and Hybridization Analyses

Primers or Probes	Nucleotide Sequence (5' to 3')	Chemistry and Location
ITS3	GCA TCG ATG AAG AAC GCA GC (SEQ ID NO:63)	5.8S rDNA universal 5' primer
ITS4	TCC TCC GCT TAT TGA TAT GC (SEQ ID NO:64)	28S rDNA universal 3' primer
ITS1	TCC GTA GGT GAA CCT GCG G (SEQ ID NO:62)	18S rDNA universal 5' primer

DNA sequencing

Primary DNA amplifications were conducted as described above. The aqueous phase of the primary PCR reaction was purified using QIAquick Spin Columns (Quiagen, Chatsworth, CA). DNA was eluted from each column with 50 µl of heat-sterilized Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Purified DNA was labeled using a dye terminator cycle sequencing kit (ABI PRISM, Perkin Elmer, Foster City, CA). One mix was made for each of the primers so that sequencing could be performed in both the forward and reverse directions. The reaction volume (20 µl) contained 9.5 µl Terminator Premix, 2 µl (1 ng) DNA template, 1 µl primer (3.2 pmol) and 7.5 µl heat-sterilized distilled H_2O. The mixture was then placed into a pre-heated (96°C) Perkin Elmer 9600 thermal cycler for 25 cycles of 96°C

for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. The PCR product was then purified before sequencing using CentriSep spin columns (Princeton Separations, Adelphia, NJ). DNA was then vacuum dried, resuspended in 6 μl of formamide-EDTA (5 μl deionized formamide plus 1 μl 50 mM EDTA, pH 8.0), and denatured for 2 min at 90°C prior to sequencing using an automated capillary DNA sequencer (ABI Systems, Model 373, Bethesda, MD).

The sequencing results were as follows:

Aspergillus flavus 5.8S ribosomal RNA gene, partial sequence, internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence.

GCTGCCCATC AAGCACGGC TTGTGTGTTG GGTCGTCGTC CCCTCTCCGG GGGGGACGGG CCCCAAAGGC AGCGGCGGCA CCGCGTCCGA TCCTCGAGCG TATGGGGCTT TGTCACCCGC TCTGTAGGCC CGGCCGGCGC TTGCCGAACG CAAATCAATC TTTTTCCAGG TTGACCTCGG ATCAGGTAGG GATACCCGCT GAACTTCAA (SEQ ID NO:1)

Aspergillus fumigatus 5.8S ribosomal RNA gene, partial sequence, internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence.

AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT TTGAACGCAC ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTCCGAG CGTCATTGCT GCCCATCAAG CACGGCTTGT GTGTTGGGCC CCCGTCCCCC TCTCCCGGGG GACGGGCCCG AAAGGCAGCG GCGGCACCGC GTCCGGTCCT CGAGCGTATG GGGCTTGTCA CCTGCTCTGT AGGCCCGGCC GGCGCCAGCC GACACCCAAC TTTATTTTTC TAAGGTTGAC CTCGGATCAG GTAGGGATAC CCGCTGAACT TAAA (SEQ ID NO:2)

Aspergillus niger 5.8S ribosomal RNA gene, partial sequence, internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence.

AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC GAAATGCGAT AACTAATGTG AATTGCAGAA

TTCAGTGAAT CATCGAGTCT TTGAACGCAC ATTGCGCCCC
CTGGTATTCC GGGGGGCATG CCTGTCCGAG CGTCATTGCT
GCCCTCAAGC ACGGCTTGTG TGTTGGGTCG CCGTCCCCCT
CTCCCGGGGG ACGGGCCCGA AAGGCAGCGG CGGCACCGCG
TCCGATCCTC GAGCGTATGG GGCTTTGTCA CCTGCTCTGT
AGGCCCGGCC GGCGCCTGCC GACGTTATCC AACCATTTTT
TTCCAGGTTG ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAA
(SEQ ID NO:3)

Aspergillus terreus 5.8S ribosomal RNA gene, partial sequence, internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence.

AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA
AGAACGCAGC GAAATGCGAT AACTAATGTG AATTGCAGAA
TTCAGTGAAT CATCGAGTCT TTGAACGCAC ATTGCGCCCC
CTGGTATTCC GGGGGGCAT GCCTGTCCGA GCGTCATTGC
TGCCCTCAAG CCCGGCTTGT GTGTTGGGCC CTCGTCCCCC
GGCTCCCGGG GGACGGGCCC GAAAGGCAGC GGCGGCACCG
CGTCCGGTCC TCGAGCGTAT GGGGCTTCGT CTTCCGCTCC
GTAGGCCCGG CCGGCGCCCG CCGAACGCAT TTATTTGCAA
CTTGTTTTTT TTTCCAGGTT GACCTCGGAT CAGGT (SEQ ID NO:4)

Aspergillus nidulans 5.8S ribosomal RNA gene, partial sequence, internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence.

AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA
AGAACGCAGC GAACTGCGAT AAGTAATGTG AATTGCAGAA
TTCAGTGAAT CATCGAGTCT TTGAACGCAC ATTGCGCCCC
CTGGCATTCC GGGGGGCATG CCTGTCCGAG CGTCATTGCT
GCCCTCAAGC CCGGCTTGTG TGTTGGGTCG TCGTCCCCCC
CCCCGGGGGA CGGGCCCGAA AGGCAGCGGC GGCACCGGTC
CGGTCCTCGA GCGTATGGGG CTTGGTCACC CGCTCGATTA
GGGCCGGCCG GGCGCCAGCC GGCGTCTCCA ACCTTATCTT
TCTCAGGTTG ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAA
(SEQ ID NO:5)

Fusarium solani (strain ATCC62877) internal transcribed spacer 2 and adjacent regions.
GAAAATGCGA TAAGTAATGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC TGGCGGGCAT GCCTGTTCGA GCGTCATTAC AACCCTCAGG CCCCCGGGCC TGGCGTTGGG GATCGGCGGA AGCCCCCTGC GGGCACAACG CCGTCCCCCA AATACAGTGG CGGTCCCGCC GCAGCTTCCA TTGCGTAGTA GCTAACACCT CGCAACTGGA GAGCGGCGCG GCCACGCCGT AAAACACCCA ACTTCTGAAT GTTGACCTCG AATCAGGTAG GAATACCCGC TGAACTTAA (SEQ ID NO:6)

Fusarium moniliforme (strain ATCC38519) internal transcribed spacer 2 and adjacent regions.
AAATGCGATA AGTAATGTGA ATTGCAAAAT TCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGC CAGTATTCTG GCGGGCATGC CTGTTCGAGC GTCATTTCAA CCCTCAAGCC CCCGGGTTTG GTGTTGGGGA TCGGCAAGCC CTTGCGGCAA GCCGGCCCCG AAATCTAGTG GCGGTCTCGC TGCAGCTTCC ATTGCGTAGT AGTAAAACCC TCGCAACTGG TACGCGGCGC GGCCAAGCCG TTAAACCCCC AACTTCTGAA TGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA (SEQ ID NO:7)

Mucor rouxii (strain ATCC24905) internal transcribed spacer 2 and adjacent regions.
AAAGTGCGAT AACTAGTGTG AATTGCATAT TCAGTGAATC ATCGAGTCTT TGAACGCAAC TTGCGCTCAT TGGTATTCCA ATGAGCACGC CTGTTTCAGT ATCAAAACAA ACCCTCTATC CAGCATTTTG TTGAATAGGA ATACTGAGAG TCTCTTGATC TATTCTGATC TCGAACCTCT TGAAATGTAC AAAGGCCTGA TCTTGTTTAA ATGCCTGAAC TTTTTTTTAA TATAAAGAGA AGCTCTTGCG GTAAACTGTG CTGGGGCCTC CCAAATAATA CTCTTTTTAA ATTTGATCTG AAATCAGGCG GGATTACCCG CTGAACTTAA (SEQ ID NO:8)

Mucor racemosus (strain ATCC22365) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AACTAGTGTG AATTGCATAT TCAGTGAATC ATCGAGTCTT TGAACGCAAC TTGCGCTCAT TGGTATTCCA ATGAGCACGC CTGTTTCAGT ATCAAAACAA ACCCTCTATC CAACTTTTGT TGTATAGGAT TATTGGGGGC CTCTCGATCT GTATAGATCT TGAAATCCCT GAAATTTACT AAGGCCTGAA CTTGTTTAAA TGCCTGAACT TTTTTTTAAT ATAAAGGAAA GCTCTTGTAA TTGACTTTGA TGGGGCCTCC CAAATAAATC TCTTTTAAAT TTGATCTGAA ATCAGGCGGG ATTACCCGCT GAACTTAA (SEQ ID NO:9)

Mucor plumbeus (strain ATCC4740) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AACTAGTGTG AATTGCATAT TCAGTGAATC ATCGAGTCTT TGAACGCAAC TTGCGCTCAT TGGTATTCCA ATGAGCACGC CTGTTTCAGT ATCAAAACAA ACCCTCTATC CAACTTTTGT TGTATAGGAT TATTGGGGGC CTCTCGATCT GTATAGATCT TGAAACCCTT GAAATTTACT AAGGCCTGAA CTTGTTTAAT GCCTGAACTT TTTTTAATA TAAAGGAAAG CTCTTGTAAT TGACTTTGAT GGGGCCTCCC AAATAAATCT TTTTTAAATT TGATCTGAAA TCAGGTGGGA TTACCCGCTG AACTTAA (SEQ ID NO:10)

Mucor indicus (strain ATCC4857) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AACTAGTGTG AATTGCATAT TCAGTGAATC ATCGAGTCTT TGAACGCATC TTGCACTCAA TGGTATTCCA TTGAGTACGC CTGTTTCAGT ATCAAAAAC AACCCTTATT CAAAATTCTT TTTTTGAATA GATATGAGTG TAGCAACCTT ACAAGTTGAG ACATTTTAAA TAAAGTCAGG CCATATCGTG GATTGAGTGC CGATACTTTT TTAATTTGA AAAGGTAAAG CATGTTGATG TCCGCTTTTT GGGCCTCCCA AATAACTTTT TAAACTTGAT CTGAAATCAG GTGGGATTAC CCGCTGAACT TAA (SEQ ID NO:11)

Mucor circinelloides f. circinelloides (strain ATCC1209B) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AACTAGTGTG AATTGCATAT TCAGTGAATC ATCGAGTCTT TGAACGCAAC TTGCGCTCAT TGGTATTCCA ATGAGCACGC CTGTTTCAGT ATCAAAACAA ACCCTCTATC CAACATTTTT GTTGAATAGG ATGACTGAGA GTCTCTTGAT CTATTCTGAT CTCGAAGCTC TTGAAATGTA CAAAGGCCTG ATCTTGTTTG AATGCCTGAA CTTTTTTTA ATATAAAGAG AAGCTCTTGC GGTAAACTGT GCTGGGGCCT CCCAAATAAC ACATCTTTAA ATTTGATCTG AAATCAGGT GGGACTACCC GCTGAACTT AA (SEQ ID NO:12)

Rhizopus oryzae (strain ATCC34965) internal transcribed spacer 2 and adjacent regions.

AGTGCGATAA CTAGTGTGAA TTGCATATTC AGTGAATCAT CGAGTCTTTG AACGCAGCTT GCACTCTATG GTTTTTCTAT AGAGTACGCC TGCTTCAGTA TCATCACAAA CCCACACATA ACATTTGTTT ATGTGGTGAT GGGTCGCATC GCTGTTTTAT TACAGTGAGC ACCTAAAATG TGTGTGATTT TCTGTCTGGC TTGCTAGGCA GGAATATTAC GCTGGTCTCA GGATCTTTTT TTTTGGTTCG CCCAGGAAGT AAAGTACAAG AGTATAATCC AGTAACTTTC AAACTATGAT CTGAAGTCAG GTGGGATTAC CCGCTGAACT TAA (SEQ ID NO:13)

Rhizopus oryzae (strain ATCC11886) internal transcribed spacer 2 and adjacent regions.

AGTGCGATAA CTAGTGTGAA TTGCATATTC AGTGAATCAT CGAGTCTTTG AACGCAGCTT GCACTCTATG GTTTTTCTAT AGAGTACGCC TGCTTCAGTA TCATCACAAA CCCACACATA ACATTTGTTT ATGTGGTAAT GGGTCGCATC GCTGTTTTAT TACAGTGAGC ACCTAAAATG TGTGTGATTT TCTGTCTGGC TTGCTAGGCA GGAATATTAC GCTGGTCTCA GGATCTTTTT CTTTGGTTCG CCCAGGAAGT AAAGTACAAG AGTATAATCC AGCAACTTTC AAACTATGAT CTGAAGTCAG GTGGGATTAC CCGCTGAACT TAA (SEQ ID NO:14)

Rhizopus microsporus (strain ATCC14056) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AACTAGTGTG AATTGCATAT TCGTGAATCA TCGAGTCTTT GAACGCAGCT TGCACTCTAT GGATCTTCTA TAGAGTACGC TTGCTTCAGT ATCATAACCA ACCCACACAT AAAATTTATT TTATGTGGTG ATGGACAAGC TCGGTTAAAT TTAATTATTA TACCGATTGT CTAAAATACA GCCTCTTTGT AATTTTCATT AAATTACGAA CTACCTAGCC ATCGTGCTTT TTTGGTCCAA CCAAAAAACA TATAATCTAG GGGTTCTGCT AGCCAGCAGA TATTTTAATG ATCTTTAACT ATGATCTGAA GTCAAGTGGG ACTACCCGCT GAACTTAA (SEQ ID NO:15)

Rhizopus microsporus (strain ATCC12276) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AACTAGTGTG AATTGCATAT TCGTGAATCA TCGAGTCTTT GAACGCAGCT TGCACTCTAT GGATCTTCTA TAGAGTACGC TTGCTTCAGT ATCATAACCA ACCCACACAT AAAATTTATT TTATGTGGTG ATGGACAAGC TCGGTTAAAT TTAATTATTA TACCGATTGT CTAAAATACA GCCTCTTTGT AATTTTCATT AAATTACGAA CTACCTAGCC ATCGTGCTTT TTTGGTCCAA CCAAAAAACA TATAATCTAG GGGTTCTGCT AGCCAGCAAA TATTTTAATG ATCTTTAACC TATGATCTGA AGTCAAGTGG GACTACCCGC TGAACTTAA (SEQ ID NO:16)

Rhizopus circinans (strain ATCC34106) internal transcribed spacer 2 and adjacent regions.

AAATTGCGAT AACTAGTGTG AATTGCATTT TCAGTGAATC ATCGAGTCTT TGAACGCAT CTTGCGCTCT TGGGATTCTT CCCTAGAGCA CACTTGCTTC AGTATCATAA CAAAACCCTC ACCTAATATT TTTTTTTTTT AAAAAAAAAA TATTAGAGTG GTATTGGGGT CTCTTTGGTA ATTCTTTGTA ATTATAAAAG TACCCTTAAA TGTCATAAAC AGGTTAGCTT TAGCTTGCCT TTAAAGATCT TCTTAGGGTA TCATTACTTT TCGTAAATCT TTAATAGGCC TGTCACATAA TTCTACCCTT AAATTTCTTA AACCTTGATC TGAAGTCAAG TGGGAGTACC CGCTGAACTT AA (SEQ ID NO:17)

Rhizopus circinans (strain ATCC34101) internal transcribed spacer 2 and adjacent regions.
AAATTGCGAT AACTAGTGTG AATTGCATTT TCAGTGAATC ATCGAGTCTT TGAACGCATC TTGCGCTCTT GGGATTCTTC CCTAGAGCAC ACTTGCTTCA GTATCATAAC AAAACCCTCA CCTAATATTT TTTTTTAAAA AAAAAAAATA TTAGAGTGGT ATTGGGGTCT CTTTGGTAAT TCTTTGTAAT TATAAAGTA CCCTTAAATG TCATAAACAG GTTAGCTTTA GCTTGCCTTT AAAGATCTTC TTAGGGTATC ATTACTTTTC GTAAATCTTT AATAGGCCTG TCACATAATT CTACCCTTAA ATTTCTTAAA CCTTGATCTG AAGTCAAGTG GGAGTACCCG CTGAACTTAA (SEQ ID NO:18)

Rhizous stolonifer (strains ATCC14037 and 6227A) internal transcribed spacer 2 and adjacent regions.
AAAGTGCGAT AACTAGTGTG AATTGCATAT TCAGTGAATC ATCGAGTCTT TGAACGCAAC TTGCACTCTA TGGTTTTCCG TAAAGTACGC TTGCTTCAGT ATCATAAAGA CCCCATCCTG ATTATTATTT TTTTATTAAA ATAATTAATT TTGGAGATAA TAAAAATGAG GCTCTTTCTT TTCTTTTTTT TTTTTTTAAA AAAAAGGGGG GGAAAGGGTC TTTTAAAATG GGCAAATTCT GGGTTTTTTA CTAAACCTGA ACTCCCCCCA AAAATTCAAA AAAAAAAAAA TGGGTTTTAC CAAATTTTTT TTTTTTTTCT CCTTTTTGTG TAGTTAATAC TCTATTAAAT TTATTTACTT GGTATTATAA CGATTATGCA AGAAGGGAGA GAACAAAGAA TAATGAAAGA GAGTTTTTAA ATAAATTCTT TTTTCATTTT TTCAATCAAT GATCTGAAGT CAAGTGGGAT TACCCGCTGA ACTTAA (SEQ ID NO:19)

Rhizomucor pusillus (strain ATCC36606) internal transcribed spacer 2 and adjacent regions.
AAATTGCGAA AAGTAATGCG ATCTGCAGCC TTTGCGAATC ATCGAATTCT CGAACGCACC TTGCACCCTT TGGTTCATCC ATTGGGTACG TCTAGTTCAG TATCTTTATT AACCCCTAAA GGTTTATTTT TTGATAAATC TTTGGATTTG CGGTGCTGAT GGATTTTCAT CCGTTCAAGC TACCCGAACA ATTTGTATGT TGTTGACCCT TGATATTTCC TTGAGGGCTT GCATTGGTAT

CTAATTTTTT ACCAGTGTGC TTCGAGATGA TCAAGTATAA
AGGTCAATCA ACCACAAATA AATTTCAACT ATGGATCTGA
ACTTAGATGG GATTACCCGC TGAACTTAA (SEQ ID NO:20)

Absidia corymbifera (strain ATCC46774) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AATTATTGCG ACTTGCATTC ATAGCGAATC
ATCGAGTTCT CGAACGCATC TTGCGCCTAG TAGTCAATCT
ACTAGGCACA GTTGTTTCAG TATCTGCAAC TACCAATCAG
TTCAACTTGG TTCTTTGAAC CTAAGCGAGC TGGAAATGGG
CTTGTGTTGA TGGCATTCAG TTGCTGTCAT GGCCTTAAAT
ACATTTAGTC CTAGGCAATT GGCTTTAGTC ATTTGCCGGA
TGTAGACTCT AGAGTGCCTG AGGAGCAACG ACTTGGTTAG
TGAGTTCATA ATTCCAAGTC AATCAGTCTC TTCTTGAACT
AGGTCTTAAT CTTTATGGAC TAGTGAGAGG ATCTAACTTG
GGTCTTCTCT TAAAACAAAC TCACATCTAG ATCTGAAATC
AACTGAGATC ACCCGCTGAA CTTAA (SEQ ID NO:21)

Absidia corymbifera (strain ATCC46773) internal transcribed spacer 2 and adjacent regions.

AAAGTGCGAT AATTATTGCG ACTTGCATTC ATAGTGAATC
ATCGAGTTCT TGAACGCATC TTGCGCCTAG TAGTCAATCT
ACTAGGCACA GTTGTTTCAG TATCTGCATC CACCAATCAA
CTTAACCTTT TGTGTTGAGT TGGAACTGGG CTTCTAGTTG
ATGGCATTTA GTTGCTGTCA TGGCCTTAAA TCAATGTCCT
AGGTGTTAGA ACATCTAACA CCGGATGGAA ACTTTAGAGC
GCTTTAAGAG CAGCTTGGTT AGTGAGTTCA ATAATTCCAA
GCATTAAGTC TTTTAATGAA CTAGCTTTTC TATCTATGGG
ACACTACTTG GAGAAATCCA AGTAACCTTT AAACTCCCAT
TTAGATCTGA AATCAACTGA GACCACCCGC TGAACTTAA (SEQ ID NO:22)

Cunninghamella elegans (strain ATCC42113) internal transcribed spacer 2 and adjacent regions.

AAATCGCGAT ATGTAATGTG ACTGCCTATA GTGAATCATC
AAATCTTTGA AACGCATCTT GCACCTTATG GTATTCCATA
AGGTACGTCT GTTTCAGTAC CACTAATAAA TCTCTCTCTA

TCCTTGATGA TAGAAAAAAA AAAAATAATT TTTACTGGGC CCGGGGAATC CTTTTTTTTT TTTAATAAAA AGGACCAATT TTGGCCCAAA AAAAAGGGTT GAACTTTTTT TACCAGATCT TGCATCTAGT AAAAACCTAG TCGGCTTTAA TAGATTTTTA TTTTCTATTA AGTTTATAGC CATTCTTATA TTTTTTAAAA TCTTGGCCTG AAATCAGATG GGATACCCGC TGAACTTAA (SEQ ID NO:23)

Pseudallescheria boydii (strain ATCC44328) internal transcribed spacer 2 and adjacent regions (teleomorph of *Scedosporium apiospermum*).
AAATGCGATA AGTAATGTAA ATTGCAAAAT TCAGTGAATC ATCGAATCTT TGAAACGCAC ATTGCGCCCG GCAGTAATCT GCCGGGCATG CCTGTCCGAG CGTCATTTCA ACCCTCGAAC CTCCGTTTC CTTAGGGAAG CCTAGGGTCG GTGTTGGGGC GCTACGGCAA GTCCTCGCAA CCCCCGTAGG CCCTGAAATA CAGTGGCGGT CCCGCCGCGG TTGCCTTCTG CGTAGTAAGT CTCTTTTGCA AGCTCGCATT GGGTCCCGGC GGAGGCCTGC CGTCAAACCA CCTAACAACT CCAGATGGTT TGACCTCGGA TCAGGTAGGG TTACCCGCTG AACTTAA (SEQ ID NO:24)

Pseudallescheria boydii (strain ATCC36282) internal transcribed spacer 2 and adjacent regions (teleomorph of *Scedosporium apiospermum*).
GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAAACGCA CATTGCGCCC GGCAGTAATC TGCCGGGCAT GCCTGTCCGA GCGTCATTTC AACCCTCGAA CCTCCGTTTC CTCAGGGAAG CTCAGGGTCG GTGTTGGGGC GCTACGGCAA GTCTTCGCAA CCCTCCGTAG GCCCTGAAAT ACAGTGGCGG TCCCGCCGCG GTTGCCTTCT GCGTAGAAGT CTCTTTTGCA AGCTCGCATT GGGTCCCGGC GGAGGCCTGC CGTCAAACCA CCTATAACTC CAAATGGTTT GACCTCGGAT CAGGTAGGGT TACCCGCTGA ACTTAA (SEQ ID NO:25)

Scedosporium apiospermum (strain ATCC64215) internal transcribed spacer 2 and adjacent regions.
GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGG CAGTAATCTG CCGGGCATGC CTGTCCGAGC GTCATTTCAA CCCTCGAACC

TCCGTTTCCT CAGGGAAGCT CAGGGTCGGT GTTGGGGCGC TACGGCGAGT CTTCGCGACC CTCCGTAGGC CCTGAAATAC AGTGGCGGTC CCGCCGCGGT TGCCTTCTGC GTAGTAAGTC TCTTTTGCAA GCTCGCATTG GGTCCCGGCG GAGGCCTGCC GTCAAACCAC CTATAACTCC AGATGGTTTG ACCTCGGATC AGGTAGGTAC CCGCTGAACT TAA (SEQ ID NO:26)

Scedosporium apiospermum (strain ATCC46173) internal transcribed spacer 2 and adjacent regions.
AAATGCGATA AGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCGG CAGTAATCTG CCGGGCATGC CTGTCCGAGC GTCATTTCAA CCCTCGAACC TCCGTTTCCT CAGGGAAGCT CAGGGTCGGT GTTGGGGCGC TACGGCGAGT CTTCGCGACC CTCCGTAGGC CCTGAAATAC AGTGGCGGTC CCGCCGCGGT TGCCTTCTGC GTAGTAAGTC TCTTTTGCAA GCTCGCATTG GGTCCCGGCG GAGGCCTGCC GTCAAACCAC CTATAACTCC AGATGGTTTG ACCTCGGATC AGGTAGGTAC CCGCTGAACT TAA (SEQ ID NO:27)

Penicillium notatum (strain ATCC10108) internal transcribed spacer 2 and adjacent regions.
AAATGCGATA CGTAATGTGA ATTGCAAATT CAGTGAATCA TCGAGTCTT TGAACGCACA TTGCGCCCCC TGGTATTCCG GGGGGCATGC CTGTCCGAGC GTCATTGCTG CCCTCAAGCA CGGCTTGTGT GTTGGGCCCC GTCCTCCGAT CCCGGGGGAC GGGCCCGAAA GGCAGCGGCG GCACCGCGTC CGGTCCTCGA GCGTATGGGG CTTTGTCACC CGCTCTGTAG GCCCGGCCGG CGCTTGCCGA TCAACCCAAA TTTTTATCCA GGTTGACCTC GGATCAGGTA GGGATACCCG CTGAACTTAA (SEQ ID NO:28)

Sporothrix schenckii (strain ATCC14284) internal transcribed spacer 2 and adjacent regions.
GAAATGCGAT ACTAATGTGA ATTGCAGAAT TCAGCGAACC ATCGAATCTT TGAACGCACA TTGCGCCCGC CAGCATTCTG GCGGGCATGC CTGTCCGAGC GTCATTTCCC CCCTCACGCG CCCCGTTGCG CGCTGGTGTT GGGGCGCCCT CCGCCTGGCG GGGGGCCCCC GAAAGCGAGT GGCGGGCCCT GTGGAAGGCT

CCGAGCGCAG TACCGAACGC ATGTTCTCCC CTCGCTCCGG AGGCCCCCCA GGCGCCCTGC CGGTGAAAAC GCGCATGACG CGCAGCTCTT TTTACAAGGT TGACCTCGGA TCAGGTGAGG ATACCCGCTG ACTTAA (SEQ ID NO:29)

Contamination precautions

Precautions were taken to avoid possible contamination of PCR samples by following the guidelines of Fujita and Kwok (13, 22). All buffers and distilled water used for PCR assays were autoclaved and fresh PCR reagents were aliquoted prior to use. Physical separation of laboratory areas used to prepare PCR assays and to analyze PCR products, and the use of aerosol-resistant pipette tips, reduced possible cross-contamination of samples by aerosols. Appropriate negative controls were included in each test run, including controls omitting either the primer or the DNA template during PCR assays.

Agarose gel electrophoresis

Gel electrophoresis was conducted in TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA, pH 8.4) at 80 V for 1 to 2 hours using gels composed of 1% (w/vol) agarose (International Technologies, New Haven, CT) and 1% (w/vol) NuSieve agar (FMC Bioproducts, Rockland, ME). Gels were stained with 0.5 µg of ethidium bromide (EtBr) per ml of distilled H_2O for 10 minutes followed by three serial washes for 10 minutes each with distilled H_2O.

Microtitration plate enzyme immunoassay for the detection of PCR products

Amplicons were detected using species-specific and genus probes labeled with digoxigenin andan all-filamentous fungal probe labeled with biotin in a streptavidin-coated microtiter plate format (13, 34). Ten µl of PCR product was added to each 1.5 ml Eppendorf tube. Single-stranded DNA was then prepared by heating the tubes at 95^{o}C for 5 minutes and cooling immediately on ice. Two-tenths of a ml of hybridization solution [4x SSC (saline sodium citrate buffer, 0.6 M NaCl, 0.06 M trisodium citrate, pH 7.0) containing 20 mM Hepes, 2 mM EDTA, and 0.15% (vol/vol) Tween 20] supplemented with 50 ng/ml each of the all-*Aspergillus* biotinylated probe and a species-specific digoxigenin-labeled probe was added to each

tube containing denatured PCR product. Tubes were mixed by inversion and placed in a water bath at 37^{o}C to allow probes to anneal to PCR product DNA. After 1 hour, 100 µl of each sample was added to duplicate wells of a commercially prepared streptavidin-coated microtitration plate (Boehringer Mannheim, Indianapolis, IN). The plate was incubated at ambient temperature for 1 hour with shaking, using a microtitration plate shaker (manufactured for Dynatech by CLTI, Middletown, NY). Plates were washed 6 times with 0.01 M potassium phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST). Each well then received 100 µl of horseradish peroxidase-conjugated, anti-digoxigenin Fab fragment (Boehringer Mannheim) diluted 1:1000 in hybridization buffer. After incubation at ambient temperature for 30 minutes with shaking, the plate was washed 6 times with PBST. One hundred µl of a mixture of one volume of 3, 3', 5, 5'-tetramethyl benzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersberg, MD) and one volume of peroxidase solution (Kirkegaard and Perry Laboratories) was added to each well and the plate was placed at ambient temperature for 10 minutes for color development. The A_{650nm} of each well was determined with a microtitration plate reader (UV Max, Molecular Devices, Inc., Menlo Park, CA). The absorbance value for the reagent blank, where DNA was absent but replaced with distilled H_2O, was subtracted from each test sample.

Statistical analysis

The Student's t test was used to determine differences between sample means. Means are expressed as the mean plus or minus the standard error from the mean. Differences were considered significant when $P<0.05$.

The following probes were used to detect and distinguish each species.

Table 2

Probe Sequences

PROBES	5' to 3' OLIGONUCLEOTIDE SEQUENCE	
Generic Biotin Probe	5' end-labeled biotinylated probe 5.8S region of rDNA	
B-58	GAA TCA TCG A(AG)T CTT TGA ACG	SEQ ID NO 61
Digoxigenin-probe	5' end-labeled digoxigenin probe ITS2 region of rDNA	
Aspergillus species		
A. flavus 22	GCA AAT CAA TCT TTT TCC	SEQ ID NO 30
A. flavus 23	GAA CGC AAA TCA ATC TTT	SEQ ID NO 31
A. fumigatus	CCG ACA CCC ATC TTT ATT	SEQ ID NO 32
A. niger	GAC GTT ATC CAA CCA TTT	SEQ ID NO 33
A. nidulans	GGC GTC TCC AAC CTT ATC	SEQ ID NO 35
A. terreus	GCA TTT ATT TGC AAC TTG	SEQ ID NO 34
Fusarium species		
F. moniliforme	TCT AGT GAC GGT CTC GCT	SEQ ID NO 49
F. oxysporum	CGT TAA TTC GCG TTC CTC	SEQ ID NO 50
F. solani	CTA ACA CCT CGC AAC TGG AGA	SEQ ID NO 51
Mucor species		
M. circinelloides	AAC ATT TTT GTG AAT AGG ATG	SEQ ID NO 39
M. indicus	CGT GGA TTG AGT GCC GAT	SEQ ID NO 38
M. plumbeus	GAA ACC CTT GAA ATT	SEQ ID NO 37
M. rouxii	GAA TAG GAA TAC TGA GAG	SEQ ID NO 36
M. racemosus	GAA ATC CCT GAA ATT	SEQ ID NO 40
Penicillium species		

Penicillium marneffei 1	GGG TTG GTC ACC ACC ATA	SEQ ID NO 47
Penicillium marneffei 2	TGG TCA CCA CCA TAT TTA	SEQ ID NO 48
Penicillium notatum	GAT CAA CCC AAA TTT TTA	SEQ ID NO 46
Rhizopus species		
R. circinans	CTT AGG GTA TCA TTA CTT	SEQ ID NO 42
R. microsporus	CAT ATA ATC TAG GGG TTC	SEQ ID NO 57
R. oryzae	GAG TAT AAT CCA G(CT)A ACT	SEQ ID NO 41
R. stolonifer	CTT GGT ATT ATA ACG ATT	SEQ ID NO 44
Rhizomucor pusillus	TCC TTG AGG GCT TGC ATT	SEQ ID NO 43
Other Genera		
Absidia corymbifera	GTT GCT GTC ATG GCC TTA	SEQ ID NO 55
Cunninghamella elegans 4	TAG TCG GCT TTA ATA GAT	SEQ ID NO 52
Cunninghamella elegans 5	TAT TAA GTT TAT AGC CAT	SEQ ID NO 53
Cunninghamella elegans 6	TAA GTT TAT AGC CAT TCT	SEQ ID NO 54
Pseudallescheria boydii	AAG TCT CTT TTG CAA GCT	SEQ ID NO 45
Sporothrix schoenckii	GAC GCG CAG CTC TTT TTA	SEQ ID NO 56
Genus Probes		
G-ASPERGILLUS	CCT CGA GCG TAT GGG GCT	SEQ ID NO 58
G-FUSARIUM	CCC AAC TTC TGA ATG TTG	SEQ ID NO 59
G-MUCOR	(AC)TG GGG CCT CCC AAA TAA	SEQ ID NO 60

Species-specific probes to the ITS2 region of rDNA for *Aspergillus fumigatus* (SEQ ID NO:32), *A. flavus* (SEQ ID NO:31), *A. niger* (SEQ ID NO:33), *A. terreus* (SEQ ID NO:34), and *A. nidulans* (SEQ ID NO:35) correctly identified each of the respective species (P<0.001), and gave no false-positive reactions with *Rhizopus*, *Mucor*, *Fusarium*, *Penicillium*, or *Candida* species. The *A. flavus* probe also recognized *A. oryzae*, which belongs to the *A. flavus* group. Identification time was reduced from a mean of 5 days by conventional methods to 8 hours.

Table 3

Aspergillus Probes

Fungus	A. fumigatus	A.nidulans	A.niger	A.terreus	A.flavus
A.fumigatus (n=6)	2.197 ± 0.187	0.002	0.000	0.001	0.001
A.nidulans (n=3)	0.001	1.315 ± 0.464	0.002	0.000	0.001
A.niger (n=5)	0.000	0.000	1.242 ± 0.471	0.001	0.003
A.terreus (n=4)	0.001	0.000	0.001	1.603 ± 0.378	0.001
A.flavus (n=6)	0.001	0.001	0.000	0.001	2.043 ± 0.390
A.oryzae (n=2)	0.001	0.002	0.001	0.001	2.445 ± 0.106
A.parasitica (n=1)	0.001	0.002	0.002	0.002	0.051
A.clavus (n=1)	0.005	0.005	0.006	0.005	0.003
C.albicans (n=1)	0.002	0.001	0.002	0.000	0.000
C.parasilosis (n=1)	0.001	0.002	0.002	0.002	0.001
C.glabrata (n=1)	0.001	0.003	0.001	0.001	0.005

C.krusei (n=1)	0.002	0.002	0.002	0.001	0.001
C.tropicalis (n=1)	0.002	0.002	0.001	0.000	0.001
F.moniliforme (n=1)	0.003	0.003	0.001	0.001	0.001
F.solani (n=1)	0.006	0.002	0.001	0.000	0.001
R.oryzae (n=1)	0.001	0.001	0.001	0.001	0.001
M.racemosus (n=1)	0.001	0.002	0.005	0.002	0.000
P.notatum (n=1)	0.001	0.002	0.002	0.002	0.000
Avg±SD negative controls	0.001 ± 0.002	0.001 ± 0.001	0.000 ± 0.002	0.000 ± 0.002	0.002 ± 0.010

Species-specific probes to the ITS2 region of rDNA for *Fusarium oxysporum*, *F. solani*, and *F. moniliforme*, correctly identified each of the respective species ($P<0.001$), and gave no false-positive reactions with *Blastomyces*, *Apophysomyces*, *Candida*, *Aspergillus*, *Mucor*, *Penecillium*, *Rhizopus*, *Rhizomucor*, *Absidia*, *Cunninghamella*, *Pseudallescheria*, *Sporothrix*, or *Neosartorya*. Empty boxes in Table 4 represent zero probe reactivity.

Table 4

Fusarium Probes

Fungus	F. oxysporum	F. solani	F. moniliforme	Generic Fusarium
F. oxysporum (n=3)	1.40 ± 0.13			1.76 ± 0.27
F. solani (n=5)		1.57 ± 0.07		1.35 ± 0.28

F. moniliforme (n=2)			1.40 ± 0.01	1.34 ± 0.91
Negative control				
Fungus	**F. oxysporum**	**F. solani**	**F. moniliforme**	**Generic Fusarium**
A.fumigatus				
A.flavus				
A.niger				
A.nidulans				
A.terreus				
A.parasiticus				
A.clavatus				
P.marneffei		0.01	0.01	
P.notatum	0.01	0.01	0.01	
Rhizopus oryzae		0.03	0.01	
Rhizopus microsporus		0.01	0.01	
Rhizopus circinans		0.01	0.01	
Rhizopus stolonifer		0.01	0.01	
Rhizomucor pusillus		0.03	0.02	
M. racemosus				
M. circinelloides				
M. rouxii				
M. plumbeus				
M. indicus				
Absidia corymbifera		0.01	0.01	
Cunninghamella elegans		0.01	0.02	
P. boydii			0.02	
Sporothrix schenckii		0.01	0.01	
C.albicans				
C.tropicalis				
C.krusei				
C.parasilosis				
C.glabrata				

Neosartorya fischeri		0.01		
Blastomyces dermatitidis				
Apophysomyces elegans				
Average of negative controls	0.001 ± 0.002	0.005 ± 0.01	0.004 ± 0.006	

Species-specific probes to various other zygomyces are presented in Table 5, showing correct identification of each species and no false positives. The exceptions are that the *M. circinelloides* probe hybridized with the *M. rouxii* DNA and the *M. plumbeus* probe hybridized with the *M. racemosus* DNA. However, the *M. rouxii* probe did not hybridize with *M. circinelloides* DNA, nor did the *M. racemosus* probe hybridize with *M. plumbeus* DNA. Therefore, by a process of elimination, each species can be correctly identified. Empty boxes in Table 5 represent zero probe reactivity.

Table 5
Zygomyces Probes

	D-probes											
FUNGUS	RORY	RMIC	RCIR	RSTOL	RPUS	MRACE	MCIR	MRX	MPLUM	MIND	ABS	CUN
R. oryzae (n=5)	1.50 ± 0.48				0.01							
R. microsporus (n=5)		0.96 ± 0.61										
R.circinans (n=3)			1.56 ± 0.19									
R. stolonifer (n=5)				2.53 ± 0.07			0.01					
Rhizomucor pusillus (n=2)					1.10 ± 0.68							
M. racemosus (n=6)				0.01		2.02 ± 0.34			0.29 ± 0.52			
M.circinelloides (n=3)							1.63 ± 0.37	0.01	0.02			
M. rouxii (n=1)							1.77	0.76				
M.plumbeus (n=2)									2.14 ± 0.25			
M. indicus (n=1)		0.01								1.70 ± 0.04		
Absidia corymbifera (n=2)					0.01				0.01		1.61 ± 0.08	
Cunninhamella elegans (n=2)		0.01										2.26 ± 0.03

Table 5 Continued

Negative control	D-probes											
FUNGUS	RORY	RMIC	RCIR	RSTOL	RPUS	MRACE	MCIR	MRX	MPLUM	MIND	ABS	CUN
A.fumigatus									0.01	0.02		
A.flavus					0.01					0.05		
A.niger								0.01				
A.nidulans									0.01	0.01		
A.terreus	0.01											
A.parasiticus					0.01					0.03		
A.clavatus										0.02		
P.marneffei			0.01									
P.notatum										0.03		
F. oxysporum								0.01				
F.solani												
F.moniliforme	0.01			0.01				0.01		0.01		
P. boydii	0.02											
Sporothrix schenckii												
C.albicans												
C.tropicalis												
C.krusei												
C.parasilosis												
C.glabrata												
Neosartorya fischeri			0.01									
Blastomyces dermatitidis												
Apophysomyces elegans												
Average	0.001 ± .004	0.001 ± 0.02	0.000 ± 0.002	0.000 ±0.003	0.001 ±0.003	0.001 ± 0.002	0.001 ±0.002	0.001 ±0.003	0.003 ±0.005	0.005 ± 0.01	0.001 ±0.001	

Species-specific probes to various other fungi are presented in Table 6, showing correct identification of each species and no false positives. Empty boxes in Table 6 represent zero probe reactivity.

Table 6

Pseudallescheria and *Sporothrix* Probes

Fungus	P. boydii	P.marneffei	P.notatum	Sporothrix schenckii
P. boydii (n=4)	1.65 ± 0.48			
P.marneffei (n=3)	0.01	1.24 ± 0.12		
P.notatum (n=3)			1.93 ± 0.25	
Sporothrix schenckii (n=3)	0.01			1.94 ± 0.25
Negative control				
Fungus	**P. boydii**	**P.marneffei**	**P.notatum**	**Sporothrix schenckii**
A.fumigatus	0.01			
A.flavus				
A.niger				
A.nidulans				
A.terreus				
A.parasiticus				
A.clavatus			0.11	
F.oxysporum		0.10		
F. solani		0.14		
F. moniliforme		0.08		
R. oryzae	0.01			
R. microsporus	0.01			
R. circinans	0.01			

R. stolonifer	0.01			
Rhizomucor pusilus				
M. racemosus		0.04		
M. circinelloides	0.01	0.09		
M. rouxii	0.01			
M. plumbeus		0.05		
M. indicus				
Absidia corymbifera	0.01			
Cunninghamela bertholletiae	0.01			
C.albicans				
C.tropicalis		0.02		
C.krusei				
C.parasilosis				
C.glabrata				
Neosatorya pseudofischeri		0.03		
Blastomyces dermatitidis	0.01			
Apophysomyces elegans	0.01			
Average Negative Controls	0 004 ± 0.002	0.013 ± 0.03	0.002 ± 0.019	0.001 ± 0.002

All of the references mentioned in this Specification are hereby incorporated by reference in their entirety.

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